Doxorubicin-Induced Id2A Gene Transcription Is Targeted at an Activating Transcription Factor/Cyclic AMP Response Element Motif through Novel Mechanisms Involving Protein Kinases Distinct from Protein Kinase C and Protein Kinase A

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We have recently shown that doxorubicin (Dox), an antineoplastic drug and an inhibitor of terminal differentiation of myogenic and adipogenic cells, induces expression of Id, a gene encoding a helix-loop-helix transcriptional inhibitor. In this study we have investigated the molecular mechanisms underlying Dox-induced Id2A expression. We have also attempted to determine whether the genetic responses to Dox are related to the UV response, a well-characterized set of reactions to UV and DNA-damaging compounds that is partly mediated by AP-1. Transient transfection of a series of deletions and point mutation derivatives of the human Id2A promoter sequence shows that two closely spaced and inverted short elements similar to an activating transcription factor (ATF) binding site or a cyclic AMP response element (CRE) are necessary and sufficient for a full response to Dox. We refer to this element as the IdATF site. Sequences containing an IdATF site conferred Dox inducibility on a minimal heterologous promoter. An electrophoretic mobility shift assay showed nuclear proteins specifically interacting with the IdATF sequence. While oligonucleotides containing either legitimate ATF/CRE or AP-1 binding sequences competed for binding, antibody supershift experiments suggested that neither CREB/ATF-1 nor AP-1 are major factors binding to IdATF. Several independent criteria suggest that Dox inducibility was independent of Ca^{2+}/phospholipid-dependent protein kinase (protein kinase C), cyclic AMP-dependent protein kinase (protein kinase A), and tyrosine kinase. Moreover, we found that Dox also induces transcription from promoters of immediate-early genes through an AP-1-independent pathway. Taken together, our results suggest that Dox elicits a novel genetic response distinct from the classical UV response.

Doxorubicin (Dox), an anthracycline anticancer drug, is most widely used for the treatment of various cancers. Despite the fact that Dox alters DNA structure by intercalation and stimulates topoisomerase II-mediated DNA cleavage (42), Dox can exert its cytotoxic effect without entering cells (62). Accumulating evidence suggests that formation of reactive free-radical species may be one of the important processes that contributes to the cytotoxic effects of the anthracyclines (51). Free-radical intermediates interact with and alter a wide variety of biomolecules, including cellular membranes and nucleic acids. Peroxidation of membranes by such intermediates alters the structure and function of lipid components that act as precursors of second messengers (63). These events would likely interfere with many inositol phospholipid-mediated signaling events, including regulation of the concentration of free cytosolic Ca^{2+} and activation of Ca^{2+}/phospholipid-dependent protein kinase (protein kinase C [PKC]) (48).

Exposure to many cytotoxic agents such as alkylating agents or antitumor quinones leads to a complex transcriptional in-

duction response known as the UV response (24), which also appears to be involved in the response to DNA damage induced by UV and ionizing radiation and whose physiological role is not yet clear. Such agents commonly lead to induction of heat shock genes (46), stimulation of differentiation of murine erythroleukemia cells (49), and induction of apoptosis (66). In addition to membrane damage, Dox treatment is also expected to result in DNA damage. However, little attention has been paid to the mechanism for the genetic response to the anthracycline class of anticancer agents.

Among the genes whose transcription is induced by DNA-damaging agents are cellular proto-oncogenes, like c-*fos* and c-*jun*, and the gene for collagenase, which all may have a role in tumor invasiveness (13, 57). Although the specific pathways responsible for transducing the signals generated by the UV response to DNA damage remain unclear, these cellular responses appear to mimic the well-described proliferative response to 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) because, like the response to TPA, activation of AP-1 in part mediates the response to DNA damage (13, 64). AP-1 is formed by combinations of the products encoded by c-*jun* and c-*fos*, and it binds to TPA response elements (TRE) present in many TPA-inducible genes (4). DNA damage engenders synthesis of AP-1 and activates preexisting AP-1 by phosphorylation or dephosphorylation. Recently it has become clear that Src tyrosine kinase, Ha-Ras, Raf-1 kinase, and mitogen-acti-

vated protein kinase may also be involved in UV-induced c-*jun* expression through an AP-1 site (14, 53). In addition, NF-κB has been shown to participate in mediating the UV response (15). In view of the potential for Dox to cause damage to both membranes and DNA, we were interested in learning how Dox alters the transcription of target genes and particularly whether Dox also alters transcription through mechanisms described for the UV response.

Clinical use of Dox is limited because of its serious cumulative-dose-dependent cardiac toxicity, which leads to irreversible degenerative cardiomyopathy (55). Our previous work demonstrated that Dox selectively inhibits the accumulation of mRNA of heart-specific genes in cardiomyocytes (29) and inhibits the transcription of muscle-specific genes in skeletal muscle cells without affecting the transcription of housekeeping genes (36). Our subsequent studies showed that Dox blocks the function of myogenic regulatory factors, at least in part because of the transcriptional induction of a dominant negative regulator, Id (38). Id can form heterodimers through its helix-loop-helix motif with E proteins (which are ubiquitously expressed basic helix-loop-helix proteins) and thus prevent myogenic regulatory factors from forming functional heterodimers with E proteins (7). On the basis of these observations, we hypothesized that induction of Id may account, at least in part, for the deleterious side effects of Dox treatment with respect to the inhibition of muscle gene transcription.

In this study, we have explored the induction mechanism of Id expression by Dox. We describe the identification of Dox response elements in the human Id2A promoter, which are similar to the consensus sequence for activation transcription factor (ATF), a member of a large family of related transcription factors that includes cyclic AMP (cAMP) response element-binding protein (CREB). We designate the sequence the IdATF sequence. A mutation in the IdATF sequence abolished nuclear-factor binding and resulted in the loss of inducible promoter activity. However, we found that ATF-1/CREB is not a major component of IdATF binding and that induced expression of IdATF site-directed transcription by Dox is mediated through kinases distinct from either PKC or the cAMP-dependent protein kinase (protein kinase A [PKA]). Furthermore, we provide several independent lines of evidence for the conclusion that Dox-induced signaling pathways are novel and do not seem to involve AP-1 activation. These findings have important implications for furthering our understanding of the molecular mechanisms of Dox-induced cardiotoxicity as well as for its cytotoxic effect on cancer cells.

MATERIALS AND METHODS

Reagents. Dox, dibutyryl cAMP, phorbol 12-myristate 13-acetate (PMA), calphostin C, staurosporine, and genistein were purchased from Sigma. *N*-[5-(Isoquinolinyl)sulfonyl]-2-methylpiperazine (H7), *N*-[2-(methylamino)ethyl]-5-isoquinoline-sulfonamide (H8), and *N*-[6-(aminohexyl)-5-chloro]-1-naphthalene-sulfonamide (W7) were obtained from Seikagaku America.

Genomic cloning and plasmid constructs. An EMBL3 library prepared from human leukocytes (Clontech) was screened with a human Id2A cDNA clone previously isolated from a human cardiac cDNA library (37). A clone (clone D-1) containing a 20-kb insert was used to obtain the human Id2A promoter region. A 3.2-kb *Sma*I DNA fragment, containing the sequence from −1.3 to +1.9 kb, was subcloned into the *Sma*I site of pBS (Stratagene) to create pBS-3.2. Digestion of pBS-3.2 with restriction endonucleases *Hin*dIII and *Nhe*I resulted in a 1.3-kb DNA fragment containing at its 5′ end restriction endonuclease cleavage sites for the enzymes *Hin*dIII, *Sph*I, *Pst*I, *Sal*I, *Xba*I, *Bam*HI, and *Sma*I (derived from the pBS polylinker) plus 1.3 kb of sequence 5′ of the mRNA start site. To create Id-1300CAT, the DNA fragment digested with *Hin*dIII and *Nhe*I was inserted into the *Hin*dIII and *Xba*I restriction endonuclease sites of pCAT-Basic (Promega). Id-834CAT was constructed by digestion of Id-1300CAT with restriction endonuclease *Sst*I, removal of the 470-bp *Sst*I DNA fragment, and recircularization by ligation of the remaining portion of the plasmid. Id-180CAT was constructed by digestion of Id-1300CAT with restriction enzymes *Hin*dIII

and *Sac*II, followed by treatment with Klenow fragment to create blunt ends and religation. A series of finer 5′ deletions were constructed by PCR. The constructs with 5′ endpoints at −152, −132, −122, −112, −102, −92, −82, and −41 were created by amplifying pBS-3.2 with the following 5′ PCR primers: Id-152, 5′-cggaagcttAACCAAGCCCACGCCC-3′; Id-132, 5′-cggaagcttCCCGCGCCCACCAATG-3′; Id-122, 5′-cggaagcttAATGGAAGCGCCCGCTCGTC-3′; Id-112, 5′-cggaagcttCGCCCGCTCGTCTTGA-3′; Id-102, 5′-cggaagcttCTTGATAGACGTGCCA-3′; Id-92, 5′-cggaagcttCGTGCCACCTTCCGCC-3′; Id-82, 5′-cggaagcttCCGCCAATGGGGACGAA-3′; and Id-41, 5′-cggaagcttGGCGAGTGCGGATAAAGC-3′. A *Hin*dIII restriction site and an extra three bases are indicated by lowercase letters. A single downstream primer with coordinates +157 to +176 was used in each of the PCR amplifications. The PCR products were digested with restriction enzymes *Hin*dIII and *Nhe*I, and then appropriate DNA fragments were gel purified and ligated into *Hin*dIII- and *Xba*I-digested pCAT-Basic. The breakpoint of each deletion derivative was determined by DNA sequencing with a synthetic 18-mer (5′-ATTTGTGAGCGGATAACA-3′) that hybridized to the plasmid region upstream of the chloramphenicol acetyltransferase (CAT)-coding region. The numeric designations of each mutant in Fig. 2 refer to the 5′ deletion endpoints defined by the primers listed above.

The reporter plasmid TRE-CAT, which contains five copies of the TRE sequence derived from the human collagenase promoter in front of the herpes simplex virus thymidine kinase gene promoter in pBLCAT2 (43), has been described (3). RSV/CREB contains the rat CREB cDNA cloned into the Rous sarcoma virus expression vector pSG5 (17). RSV/KCREB is an expression plasmid for KCREB which contains a mutation of a single amino acid in the DNA binding domain of CREB (65). MAK is the expression plasmid for mouse PKA (31). Serum response element (SRE)-CAT and CRE-CAT have been described (30). −73Col-CAT, which contains DNA sequences from −73 to +63 of the human collagenase gene promoter, has been described (1). −1.1/+740*jun*CAT, −132/+170*jun*CAT, and −132/+170ΔTRE*jun*CAT have been described (2). pJC6 and pJTX contain c-*jun* promoter sequences from −225 to +150 without or with a mutation of TRE, respectively (22). c-*fos*-CAT contains 2 kb of the 5′-flanking region of the c-*fos* gene (16). *zif268CAT* contains 2.5 kb of the 5′-flanking region of the *zif268* gene (12). G4-CREB (8), which contains a fusion of the Gal4 DNA binding domain and CREB, and G5BCAT (41) have been described.

Site-directed mutagenesis of the Id2A promoter by PCR. Introduction of site-specific mutations and deletions into the Id2A promoter fragment was performed by a PCR-based method as previously described (27). Briefly, for each mutation a pair of overlapping primers containing the required mutations were used in two separate PCRs to produce two DNA fragments in the region to be mutated. The fragments were gel purified, and 100 ng of these primary PCR fragments was used in a further PCR together with outside primers containing a *Hin*dIII restriction site at the 5′ end and an *Nhe*I restriction site at the 3′ end to produce a full-length DNA fragment containing the appropriate mutations. These PCR products were digested with *Hin*dIII and *Nhe*I, gel purified, and cloned into the *Hin*dIII and *Xba*I sites of pCAT-Basic. Mutated sequences were confirmed by sequencing. Oligonucleotides for introducing mutations were as follows, with mutated bases in lowercase letters: oligonucleotide μ1, 5′-GCCCCGCGCGaGCtCCCACC-3′; oligonucleotide μ2, 5′-AATGGAAGCGCgaGCTCGTC-3′; oligonucleotide μ3, 5′-TTGATAGAGcTcCCACCTTC-3′; oligonucleotide μ4, 5′-GGGGACGAgctcAAGCTCCA-3′; and oligonucleotide μ5, 5′-CGTGTGGagCtcGCGAGTGC-3′. Each of the mutagenic oligonucleotides contained a *Sac*I restriction site which allowed selection of clones that contained the mutation. Id-150(−131μ, −109μ), carrying mutations at both −131 and −109, was made from μ1. Id-150(−109μ, −92μ), carrying mutations at both −109 and −92, was made from μ2. Id-150(−65μ, −47μ), carrying mutations at both −65 and −47, was made from μ4. The mutations these clones carry are the sum of the mutations in μ1 and μ2, μ2 and μ3, and μ4 and μ5, respectively.

Internal deletion constructs μ7, μ8, and μ9 were made from Id-150(−131μ, −109μ), Id-150(−109μ, −92μ), and Id-150(−65μ, −47μ), respectively. Those parental plasmids were digested with the restriction endonuclease *Sac*I. The small *Sst*I fragments were removed by gel electrophoresis, and the purified plasmids were recircularized through their cohesive *Sst*I ends.

Plasmid μ6 was constructed by digesting μ2 with *Sst*I. The *Sst*I site was then treated with Klenow fragment without deoxynucleoside triphosphates to create blunt ends and was recircularized with T4 DNA ligase. The resulting construct does not contain the 5 bp present in the wild-type gene between −109 and −105.

Heterologous promoter fusion. Double-stranded oligonucleotides containing DNA sequences between −126 and −85 with *Sac*I and *Kpn*I ends, and mutant versions of this sequence, were ligated into the *Sac*I and *Kpn*I sites of ΔIL3CAT (31) to generate wtATF-CAT and μATF-CAT, respectively. ΔIL3CAT contains basal promoter sequences (−50 to +30) of the mouse interleukin 3 IL-3 gene linked to CAT coding sequences (30).

Cell culture, DNA transfection, and CAT assay. HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS). Unless otherwise specified, each 100-mm-diameter culture dish was incubated for 20 h with a calcium phosphate-DNA coprecipitate containing 10 μg of reporter plasmids and 5 μg of an expression vector. After removal of excess DNA, cells were kept in fresh medium containing 10% FBS, and the reagents specified in the figure legends were added to the medium. After 20 h of treatment with reagents, cell extracts were prepared and ~100 μg of extract protein

was analyzed for CAT activity as described previously (44). In experiments involving protein kinase inhibitors, various amounts of inhibitors were added 15 min before Dox treatment. CAT activity was normalized to protein amounts which were determined by a Bio-Rad protein assay. All transfections were performed in duplicate with at least two separate preparations of each plasmid.

Oligonucleotide and EMSA. The sequences of the oligonucleotides used as probes or competitors in electrophoretic mobility shift assays (EMSAs) were as follows, with a core motif underlined and mutated bases in lowercase letter (only one strand is shown): oligonucleotide A, 5′-CGCCC<u>GCTCGTC</u>TTGATA; oligonucleotide B, 5′-TTGAT<u>AGACGTGC</u>CACCT; oligonucleotide Aμ, 5′-CG CCC<u>GagCtc</u>CTTGATA; oligonucleotide Bμ, 5′-TTGAT<u>AGagcTc</u>CACCT; oligo(−113/−74), 5′-GCGCCC<u>GCTCGTC</u>TTGAT<u>AGACGTGC</u>CACCTTCC GCCAATG; ATF/CRE, 5′-AGAGATTGCC<u>TGACGTCA</u>GAGAGCTAG; AP-1, 5′-CGCTTGA<u>TGAGTCA</u>GCCGGAA; Sp-1, 5-GACCCTCG<u>CCCCAC CCC</u>ATCCCCT; AP-2, 5′-GATCGAACTGACC<u>GCCCGCGGCC</u>CGT-3′; and NF-κB, 5′-AGTTGAG<u>GGGACTTTCC</u>CAGGC-3′.

Nuclear extracts from HeLa cells were prepared as previously described (18). Sequences for oligonucleotides A and B were derived from the human Id2A gene promoter spanning from −112 to −95 and from −100 to −83, respectively. Sequences for the ATF/CRE and AP-1 oligonucleotides were derived from the rat somatostatin gene (45) and the human collagenase gene (1), respectively. The sequence for the Sp-1 oligonucleotide was derived from the human skeletal α-actin promoter (61). The sequence for AP-2 and NF-κB oligonucleotides were derived from the human metallothionein IIA gene (67) and the mouse immunoglobulin κ light-chain gene (5), respectively. All probes were 5′ end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP (>5,000 Ci/mmol; Amersham). Binding reactions were performed for 20 min at room temperature with 10 to 15 μg of total protein in 25 μl of a solution containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 15% glycerol, 2 μg of poly(dI-dC), 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and about 10,000 cpm of ^{32}P-labeled oligonucleotides. DNA-protein complexes were separated from unbound DNA probe on nondenaturing 6% polyacrylamide gels. For the competition experiments, unless otherwise indicated, 0.5 ng of the labeled oligonucleotide was mixed with 50 ng of unlabeled competitor oligonucleotides prior to the addition of proteins. In supershift experiments, 2 μl of antibodies against Sp-1, CREB, or ATF-1 (Santa Cruz Biotechnology) was preincubated with nuclear extracts for 30 min at 25°C. ^{32}P-labeled probe was then added, and the incubation was continued for an additional 20 min before electrophoretic separation.

Northern (RNA) blot analyses. RNA was prepared by the acid guanidinium-phenol chloroform extraction procedure (11). Total RNA (20 μg) was fractionated on a 1.2%-agarose-formaldehyde gel. After transfer, the nylon membranes (Hybond N+; Amersham) were prehybridized and then hybridized with randomly primed ^{32}P-labeled cDNA probes for *zif268*, c-*jun*, and α-tubulin or ^{32}P-labeled cRNA probe for Id2 as described previously. Blots were washed for 20 min at 55°C in 0.1× SSC–0.1% sodium dodecyl sulfate (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), dried, and subjected to autoradiography.

RESULTS

Inducible expression of the Id2A-CAT fusion gene. We have recently shown that there are two genes coding for Id2 in the human genome, Id2A and Id2B (37). Sequence analyses of the Id2B cDNA and genomic clones suggest that the Id2B gene is a transcribed pseudogene which codes for a nonfunctional truncated form of the Id2 protein. Thus, we focused on the characterization of the Id2A promoter to understand the molecular details of how Id2 expression is controlled in the presence and absence of Dox. In this study, we constructed chimeric plasmids in which various fragments of the 5′-flanking region of the human Id2A gene were fused to the bacterial CAT reporter gene. Recently we have shown that Id-1300 CAT, which contains 1.3 kb of Id2A 5′-flanking region, is capable of mediating Dox-inducible expression in 10T1/2 cells (38). Among several cell lines tested, including C2, HeLa, 10T1/2, and L8 cells, HeLa cells showed the highest degree of inducibility by Dox. The induction of activity of Id-1300CAT was approximately sixfold in HeLa cells and approximately threefold in 10T1/2, L8, and C2 cells (data not shown). We therefore chose the HeLa cell environment as best suited to narrow down the promoter region required for inducible expression of the Id2A gene by Dox. To determine whether HeLa cells are germane to our study of the inducible expression of the Id2A gene by Dox, we performed Northern blot analysis. Northern blots, containing total RNA made from subconfluent cultures of HeLa cells that were exposed for 2 h
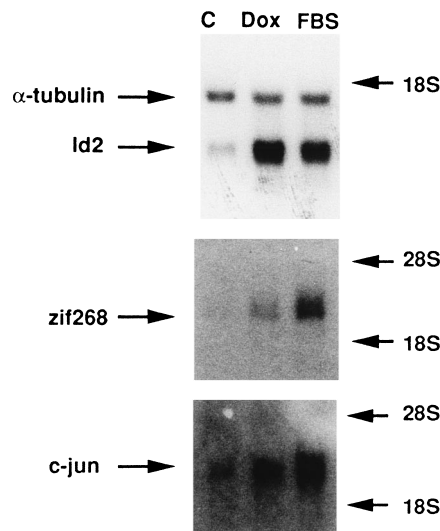

FIG. 1. Id2 gene and immediate-early gene induction by Dox and serum. Northern blots were prepared from total RNAs made from subconfluent HeLa cells exposed to medium alone (C), 1 μM Dox, or 20% FBS for 2 h and probed with radiolabeled c-*jun*, *zif268*, and α-tubulin cDNAs and with radioactive cRNA for Id2.

either to 1 μM Dox or to 20% FBS as a control, were prepared. The blots were then hybridized to the probes made from Id2 cDNA and α-tubulin cDNA. Dox increased the Id2 mRNA level in HeLa cells (about eightfold) to a level which was almost comparable to the level of induction observed with FBS stimulation (Fig. 1). Dox also induces the endogenous mRNA levels for *zif268* and c-*jun* (Fig. 1). The mRNA levels for α-tubulin were unaltered by either Dox or FBS treatment, suggesting that induction in response to Dox and FBS is gene specific.

Analysis of a series of reporter gene mutants in HeLa cells revealed a significant level of inducibility (4.3- to 6.3 fold) by Dox despite deletion of the sequence from −1300 to −152, whereas further deletion to −92 eliminated inducibility (Fig. 2). The Id-92CAT construct continues to exhibit core promoter activity, since further deletion of sequences to position −41 dropped basal expression to a background level. These results indicate that the 5′ boundaries of the sequences responsible for Dox-induced expression of the Id2A gene are located between −152 and −92. To map the Dox response element(s) precisely in this region, we made four finer deletion constructs with 5′ ends at −132 −122, −112, and −102. Each of these mutants had the same 3′ endpoint at +30. Transient-transfection analysis of these mutant constructs showed that a promoter fragment covering −112 to +30 mediated virtually the same inducibility as the 1.3-kb promoter fragment. Deletion to position −102 resulted in the reduction of inducibility, and further deletion to −92 completely abolished the inducibility. Further deletion to −41 and beyond rendered the promoter inactive. These data place the 5′ boundaries of the minimal promoter for strong induction by Dox at −112 and the boundaries for moderate activation at −102. These results indicate that the 5′ border of a regulatory element required for full Dox inducibility is located between −112 and −92 of the Id2A promoter. Close inspection of this region revealed two closely spaced and inverted short elements, from −95 to −88 (5′-AG ACGTGC-3′) and from −107 to −100 (5′-AGACGAGC-3′, complementary strand) (Fig. 3A). A search among known transcription activator binding sites similar to these short ele-
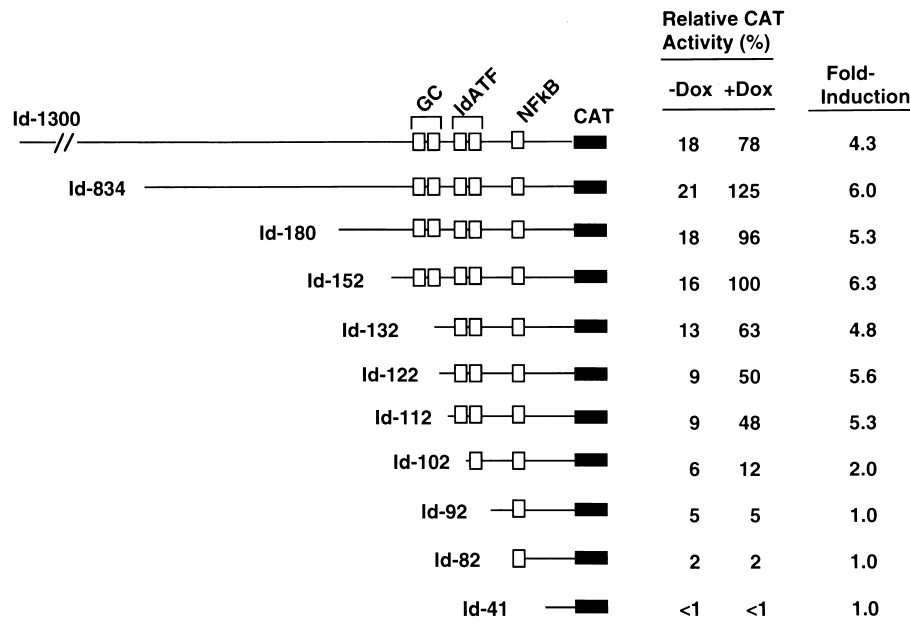
FIG. 2. 5′ deletion analysis of the human Id2A promoter. A schematic diagram of the structure of each deletion CAT reporter construct is shown. The locations of putative nuclear protein binding sites, the GC box, IdATF sites, and NF-κB-like sites are indicated. The solid line indicates the portion of the 5′-flanking region that is included in the construct. All constructs end at position +30. Deletion mutants with their 5′ endpoints are indicated. HeLa cells were transfected with each construct and then were treated with 1 μM Dox for 20 h before extract preparation or were left untreated. CAT activities were measured and are expressed relative to that of Id-152CAT with Dox treatment. Data are the means of three to six independent experiments with different preparations of plasmid DNA.

ments revealed identities of 7 of 8 bp and 6 of 8 bp, respectively, with the nonpalindromic ATF/CRE site (5′-TGACG TGC-3′) that has been recently described for the transforming growth factor β2 gene promoter (34). Thus, we henceforth refer to these sequences as IdATF sites.
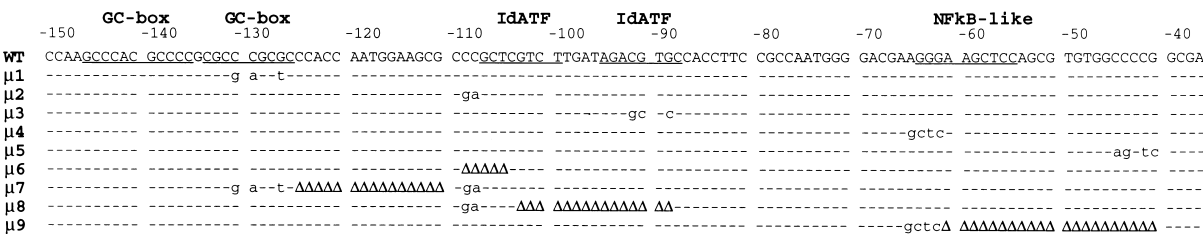
Site-directed mutagenesis in the Id-152CAT context defines sequences required for Dox inducibility. To test directly the role of the sequences spanning between −112 and −92, overlap PCR mutagenesis was used to modify individually or in combination the two IdATF sites present in this region (Fig. 3A). Compared with Dox, which showed a >6-fold induction of expression by the wild-type reporter, the μ3 construct, which contains mutations at the IdATF site located at −95, showed a markedly reduced level of induction by Dox (~1.9-fold [Fig. 3C]). The μ6 construct, which contains mutations at the IdATF site located at −107, also showed a moderately reduced level of induction by Dox (to about threefold). Thus, mutation of either IdATF sequence reduces the level of induction by Dox. A construct (μ8) that carries both sets of mutations totally failed to respond to Dox, whereas the uninduced level of this construct was still about four times higher than that of Id-82CAT (data not shown), indicating that the μ8 construct is transcriptionally competent. In contrast, neither mutation nor deletion of a GC box (μ1, μ2, μ5, and μ7) or an NF-κB-like site (μ4 and μ9) had an effect on either uninduced or Dox-induced expression (Fig. 3B and C). These data indicate that each IdATF site contributes to Dox-induced activation but that neither site has a major effect on the uninduced expression of Id-152CAT. Furthermore, these results show that only one IdATF site is necessary for directing measurable Dox-inducible expression and that the >6-fold induction of CAT activity by Dox observed in the Id-152CAT construct is fully attributable to the short region encompassing two IdATF sites.

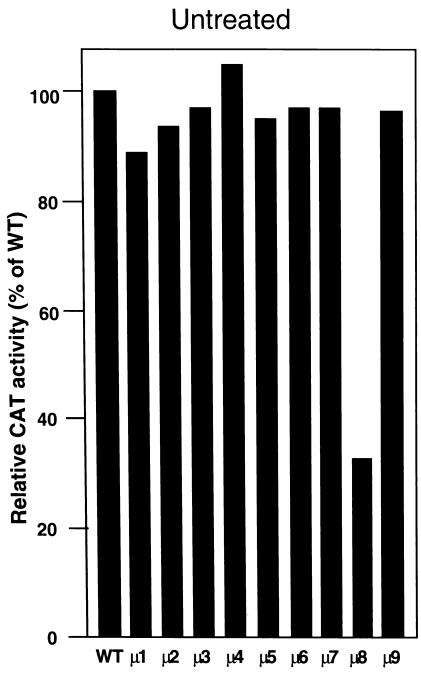**IdATF sequences are able to confer Dox-induced expression on a heterologous promoter.** To address the question of whether the promoter region containing two IdATF sites can autonomously confer responsiveness to Dox, or whether cooperation with the specific basal promoter elements of the Id2A promoter is required, we tested a double-stranded oligonucleotide (wtATF) extending from −126 to −85 for its ability to confer activation by Dox on a heterologous promoter. The ΔIL3CAT construct, which contains a minimal promoter region of the mouse IL-3 gene, did not respond to Dox (data not shown). Insertion of wtATF sequences immediately upstream of this minimal promoter sequence rendered the ΔIL3CAT construct responsive to Dox (Fig. 4). In contrast, μATF-CAT, which contains mutated sequences at both of the two IdATF sites, was not Dox inducible. These data show that promoter sequences containing IdATF sites are able to confer Dox-induced expression on a heterologous promoter.

Identification of nuclear proteins binding to the IdATF site. EMSAs identified DNA-binding proteins that interact with the IdATF sites. Nuclear extracts from HeLa cells were incubated with end-labeled oligonucleotide A, which contains the proximal IdATF site, and then subjected to gel electrophoresis. The interaction of the oligonucleotide A probe with HeLa cell nuclear extracts resulted in two specific protein-DNA complexes (C-I and C-II [Fig. 5A]). Specificity of binding was demonstrated by competition assays. Competition with a 100-fold excess of either unlabeled oligonucleotide A or B, which contains the distal IdATF site, completely eliminated the C-I and C-II complexes, whereas virtually no competition was observed with a 100-fold molar excess of unlabeled oligonucleotides Aμ and Bμ, which contain mutations at each IdATF site. These results suggest that C-I and C-II are sequence-specific complexes. Two other complexes (Fig. 5A) appear to be nonspecific, because these are not eliminated completely even in the presence of a 400-fold molar excess of unlabeled oligonucleotides A and B, and complexes with the same migration as these two bands were observed when either Aμ or Bμ was used
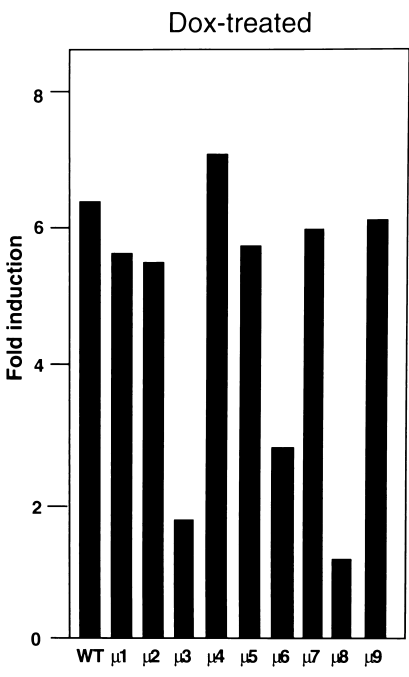
A


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              GC-box        GC-box                     IdATF       IdATF                      NFkB-like
      -150         -140         -130         -120         -110         -100          -90          -80          -70          -60          -50          -40
WT    CCAAGCCCAC GCCCCGCGCC CGCGCCCACC AATGGAAGCG CCCGCTCGTC TTGATAGACG TGCCACCTTC CGCCAATGGG GACGAAGGGA AGCTCCAGCG TGTGGCCCCG GCGA
µ1    ---------- -------g a--t----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----
µ2    ---------- ---------- ---------- ---------- -ga------- ---------- ---------- ---------- ---------- ---------- ---------- ----
µ3    ---------- ---------- ---------- ---------- ---------- -------gc -c------- ---------- ---------- ---------- ---------- ----
µ4    ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----gctc- ---------- ---------- ---------- ----
µ5    ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----ag-tc ----
µ6    ---------- ---------- ---------- ---------- -ΔΔΔΔΔ---- ---------- ---------- ---------- ---------- ---------- ---------- ----
µ7    ---------- -------g a--t-ΔΔΔΔΔ ΔΔΔΔΔΔΔΔΔΔ -ga------- ---------- ---------- ---------- ---------- ---------- ---------- ----
µ8    ---------- ---------- ---------- ---------- -ga----ΔΔΔ ΔΔΔΔΔΔΔΔΔΔ ΔΔ------- ---------- ---------- ---------- ---------- ----
µ9    ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----gctcΔ ΔΔΔΔΔΔΔΔΔΔ ΔΔΔΔΔΔΔΔΔΔ ----
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B   **C**

FIG. 3. Site-specific mutations of the Id2A promoter. (A) Sequences of site-specific mutations (µ1 to µ9) are in lowercase letters below the corresponding promoter sequences; identity to the wild-type (WT) sequence is denoted by a dash. The GC box, IdATF, and NF-κB-like sites are underlined. Δ, deleted nucleotides. (B) Promoter activity in untreated HeLa cells. HeLa cells were transfected with each reporter construct indicated in Fig. 2A. CAT activities were measured and are expressed relative to that of wild-type (WT) plasmid, in which the CAT gene is under the control of the wild-type Id2A promoter sequences spanning from −152 to +30. (C) Fold induction of promoter activity by Dox treatment. HeLa cells were transfected with each reporter construct and were treated with 1 µM Dox for 20 h. The CAT activity of each construct is expressed as the fold increase over the uninduced level of CAT activity of each construct. Data are the means of three to six independent experiments with different preparations of plasmid DNA.

as a probe (data not shown). Identical results were obtained when EMSA studies were performed with oligonucleotide B as the probe and with the same set of competitors (data not shown). It is worth stressing that the nucleotide sequence altered by mutagenesis was the same as those used in the functional analysis by CAT assays described above. Binding of complexes was also specifically competed for by either bona fide ATF/CRE or AP-1 sequences (Fig. 5A), while unrelated Sp-1, AP-2, and NF-κB oligonucleotides did not compete for binding. These data demonstrate that the specific proteins that make up the C-I and C-II complexes are capable of binding to either legitimate ATF/CRE or AP-1 binding sites. Competition by both ATF/CRE and AP-1 binding sites is not surprising, given that these sequences differ by only one base (see Materials and Methods). To determine whether either CREB or ATF-1 is actually present in the complex identified here, we

tested whether antibodies directed against these factors could perturb the complex. Anti-Sp-1 antibody was used as a negative control. Formation of the complex was not affected by any of these polyclonal antisera (Fig. 5A). In an experiment performed in parallel, antisera directed against either CREB or ATF-1 were able to supershift complexes formed by the interaction between the bona fide ATF/CRE probe and HeLa cell nuclear extract (Fig. 5A). We conclude that neither CREB nor ATF-1 is a significant component of complexes that form with the IdATF probe. Similarly, participation of either c-Fos or c-Jun proteins in forming C-I and C-II complexes is unlikely, because antisera directed against either c-Fos or c-Jun did not affect the complexes (data not shown).

In view of the facts that two IdATF sites are necessary for a full response of the Id2 promoter to Dox (Fig. 3) and that sequences between −126 and −85 (which encompass both
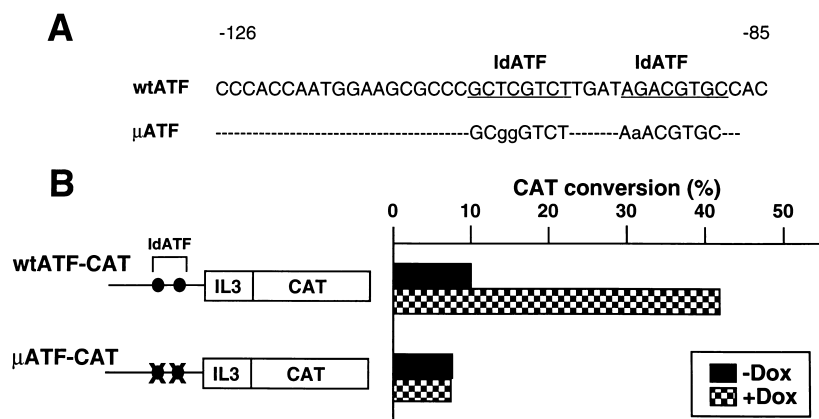
FIG. 4. Effects of IdATF sites on a heterologous promoter. (A) Double-stranded oligonucleotides containing either the wild-type sequences of the Id2A promoter between −126 to −85 (wtATF) or a mutated version (μATF) at the IdATF sites (mutated bases are indicated by lowercase letters) were inserted into the ΔIL3CAT reporter plasmid to yield wtATF-CAT and μATF-CAT, respectively. (B) HeLa cells were transfected with one of these constructs and then treated with 1 μM Dox for 20 h or left untreated. The results of a representative experiment is shown. Comparable results were obtained in three independent experiments.

oligonucleotides A and B) confer Dox inducibility to a heterologous promoter (Fig. 4), we next determined whether there is any recognition site(s) for nuclear proteins other than IdATF sites within the sequences containing the two IdATF sites. EMSA studies using an oligonucleotide containing both ATF sites [referred to as oligo(−113/−74)] revealed two shifted complexes which were both efficiently competed for by either unlabeled oligonucleotide A or B (Fig. 5B), indicating that nuclear proteins present in the shifted complexes formed with oligo(−113/−74) probe specifically bind only to the IdATF sites. These data, along with the functional analyses shown in Fig. 3 and 4, further support our previous conclusion that IdATF sites and their cognate DNA-binding proteins mediate the effects of Dox.

We compared the IdATF site-binding activities of control and Dox-treated HeLa cells. No visible changes were seen by EMSA (data not shown). These results suggest that induction of the Id2A promoter by Dox is not due to a change in the DNA-binding activities of nuclear proteins recognizing the IdATF site.

Involvement of kinases distinct from PKC in activation of the Id2A promoter by Dox. Since protein kinases are key components of many signaling pathways in eukaryotic cells (28), we tested the effects on Dox inducibility of a set of different protein kinase inhibitors, including H7, H8, calphostin C, staurosporine, genistein, and W7. HeLa cells transfected with Id-152CAT were exposed to each inhibitor 15 min prior to Dox treatment. The results are summarized in Fig. 6. A general inhibitor of protein kinases, H7 (30 μM), almost completely inhibited induction of Id2A promoter activity by Dox. The inhibitory effect of H7 was not due to nonspecific toxicity inhibiting general transcription, since uninduced levels of expression of Id-152CAT and pSV2CAT were not affected by H7 treatment (data not shown). By contrast, a tyrosine kinase inhibitor, genistein, at a concentration (1 μM) which fully blocks the induction of c-*jun* by UV in HeLa S3 cells (14), had no effect on Dox induction of transcription (Fig. 6). Likewise, a Ca^{2+}/calmodulin-dependent kinase inhibitor, W7 (20 μM), had a minimal effect on the inducibility of Id-152CAT. Particularly, a general inhibitor of protein kinases, staurosporine (2 nM), had no effect on Dox inducibility. We chose the concentration of 2 nM because staurosporine has a strong cytotoxic effect, with 50% inhibitory concentrations (IC$_{50}$s) of less than 3 nM for HeLa S3 cells (60), and it is likely that staurosporine

inhibits PKC (IC$_{50}$ = 2.7 nM) but not cyclic-nucleotide-dependent kinases (IC$_{50}$ = 8.2 nM) or pp60 v-Src tyrosine kinase (IC$_{50}$ = 6.4 nM) at this low concentration (47). Indeed, 2 nM staurosporine completely inhibited the induction of SRE-CAT by PMA, a direct activator of PKC (data not shown). A potent inhibitor of cyclic-nucleotide-dependent protein kinase, H8 (26), moderately reduced the level of Dox induction (to about 50%), and a PKC-specific inhibitor, calphostin C (59), only minimally reduced the level of induction. As expected, induction of CRE-CAT by cAMP was also completely blocked by H8 and induction of SRE-CAT activity by PMA was effectively inhibited by calphostin C (data not shown).

These inhibitor experiments suggest that PKC is not a major kinase that mediates the Dox response of the Id2A promoter. To further distinguish between Dox-induced signals and the response to PKC activation by PMA, we attempted to specifically block the PKC pathway by using a negative dominant expression vector. The expression vector (Raf-C4), which encodes a carboxyl-terminal deletion mutant of c-Raf-1, acts as a negative dominant (10). c-Raf-1 is a required kinase in the PKC- and Ras-signaling pathways (10). Cotransfection of Raf-C4 completely blocked PMA-induced activation of SRE-CAT, while Dox-induced activation of Id-152CAT was only weakly effected (Fig. 7A). These data, along with the characterization of Dox induction mechanisms by using protein kinase inhibitors, indicate that Dox and PMA activate different signaling pathways. In addition, since Raf-1 kinase has been shown to be obligatory in the UV response (14, 53), as well as in the PMA response, our results are consistent with the notion that the genetic response to Dox is distinct from the UV response.

PKA and CREB also do not mediate the Dox response. The function of CREB to induce gene expression by binding to a CRE is regulated primarily by PKA-mediated phosphorylation. Such phosphorylation steps are dependent, in turn, on cAMP and can be blocked by agents, such as H8, that are specific for cyclic-nucleotide-dependent kinases. Since H8 moderately inhibits the Dox inducibility of the Id2A promoter, we examined the potential involvement in Dox induction of CREB-dependent transcriptional activation through PKA-mediated phosphorylation. Because CREB belongs to a growing family of transcription factors that contain highly conserved domains necessary for both protein-protein interactions and DNA binding (9, 39), it is possible to interfere with its action
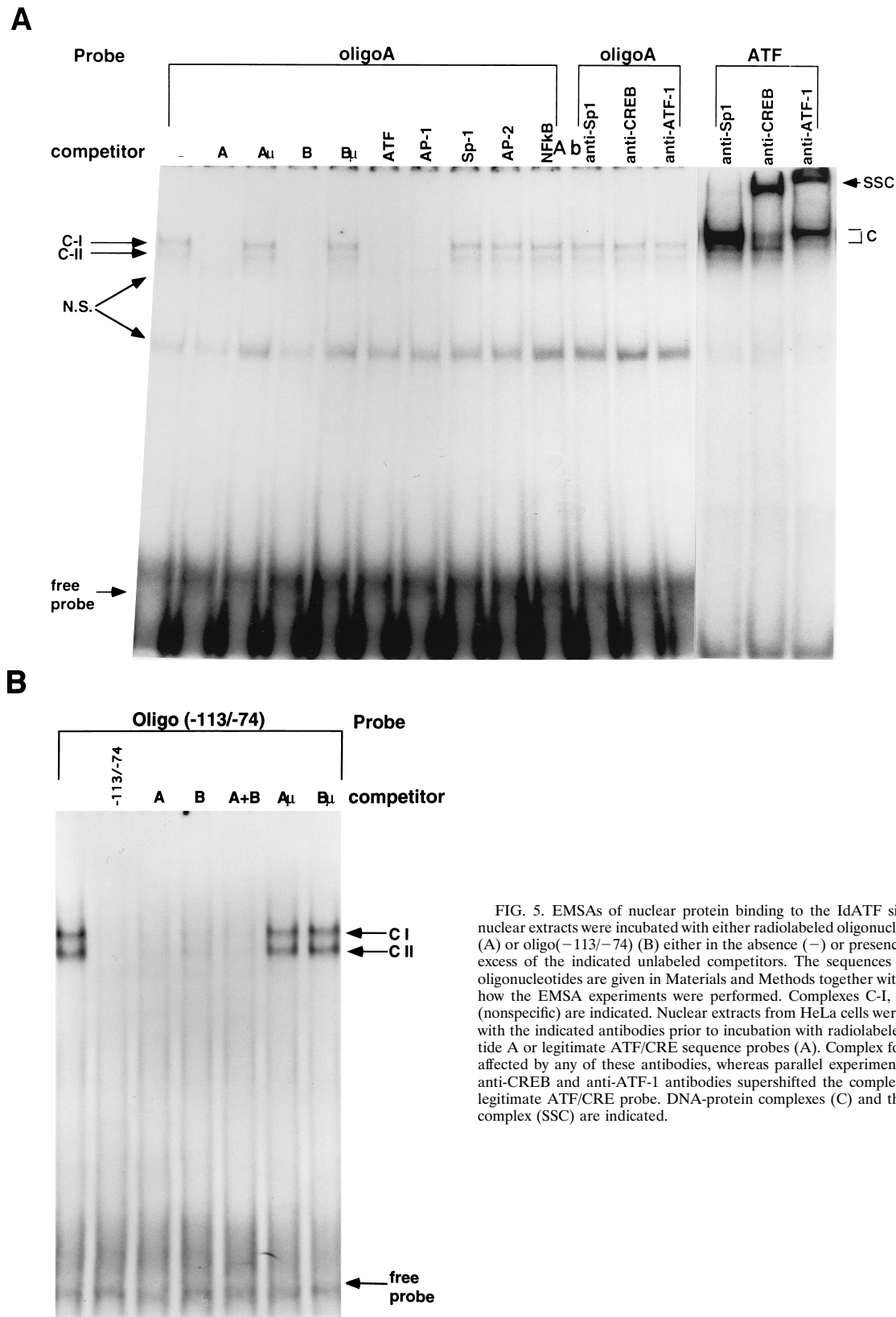
A

B

FIG. 5. EMSAs of nuclear protein binding to the IdATF sites. HeLa cell nuclear extracts were incubated with either radiolabeled oligonucleotide A probe (A) or oligo(−113/−74) (B) either in the absence (−) or presence of a 100-fold excess of the indicated unlabeled competitors. The sequences of the various oligonucleotides are given in Materials and Methods together with the details of how the EMSA experiments were performed. Complexes C-I, C-II, and N.S. (nonspecific) are indicated. Nuclear extracts from HeLa cells were preincubated with the indicated antibodies prior to incubation with radiolabeled oligonucleotide A or legitimate ATF/CRE sequence probes (A). Complex formation is not affected by any of these antibodies, whereas parallel experiments showed that anti-CREB and anti-ATF-1 antibodies supershifted the complex formed on a legitimate ATF/CRE probe. DNA-protein complexes (C) and the supershifted complex (SSC) are indicated.
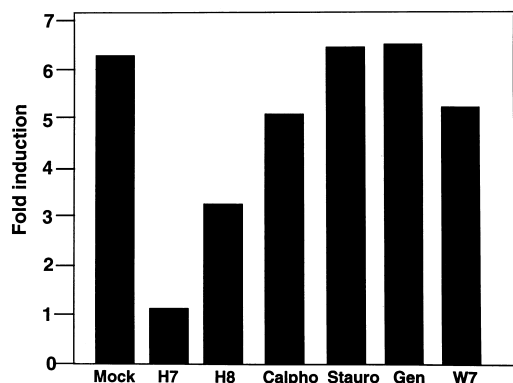
FIG. 6. Effects of protein kinase inhibitors on Dox inducibility. Twenty hours after HeLa cells were transfected with 10 μg of Id-152CAT, solvent alone (Mock [dimethyl sulfoxide at 0.1%]), H7 (30 μM), H8 (30 μM), calphostin C (Calpo; 1 μM), staurosporine (Stauro; 2 nM), genistein (Gen; 1 μM), and W7 (20 μM) were added to the culture medium 15 min prior to treatment with Dox (1 μM). After 20 h of treatment, cells were harvested and CAT activities were measured. CAT activities are expressed as the fold increase over the activity without Dox treatment. Comparable results were obtained in three independent experiments.

by expressing a mutant form of CREB that can dimerize with the wild-type factor but cannot activate transcription. We tested one such mutant, KCREB, which contains a single point mutation in its DNA binding domain and dominantly inhibits cAMP-mediated CRE-CAT activation (65). Although the precise mechanisms by which KCREB functions are not completely established, one possible explanation is that a single functional DNA binding domain of a KCREB and wild-type CREB heterodimer lacks activity when bound to the promoter. Overexpression of KCREB had no effect on Dox-induced activation of Id-152CAT, whereas activation of CRE-CAT by cAMP was efficiently blocked by KCREB (Fig. 7A). Likewise, overexpression of CREMε repressor proteins that antagonize CREB-dependent transcriptional activation by competitive binding to the CRE site (8) had no effect on Dox-induced enhancement of Id2A promoter activity (data not shown).

To further demonstrate that Dox does not activate signaling pathways involving PKA, we determined whether Dox treatment allows induction of transcription of G5BCAT by a fusion gene (G4-CREB) encoding the *GAL4* DNA binding domain and rat CREB (Fig. 7B). In response to cAMP elevation, the catalytic subunit of PKA dissociates from the regulatory subunit and migrates into the nucleus, where it phosphorylates CREB (9). Cotransfection of MAK, the catalytic subunit of mouse PKA, with G4-CREB resulted in a strong stimulation of G5BCAT reporter activity; this stimulation is consistent with previously published results (8). cAMP treatment had the same effects as MAK transfection. Since the presence of the Gal4 DNA binding domain alone does not induce G5BCAT expression regardless of the cotransfection of a PKA expression vector or cAMP treatment (data not shown), we attributed the enhanced CAT expression of G5BCAT to increased CREB transactivation by PKA or cAMP. In contrast to cAMP, Dox treatment had no effect on CAT expression from G5BCAT. These results suggest that Dox-induced pathways do not overlap with the cAMP-PKA-CREB signaling pathway.

Dox induces immediate-early gene expression independent of TRE sites. Many DNA-damaging agents have been shown to activate expression of immediate-early genes through *cis*-regulatory elements required for TPA-inducible expression (24). To examine whether any TPA-inducible genes are also responsive to Dox, we transfected HeLa cells with CAT reporter

plasmids containing the promoter sequences of various TPA-inducible genes and measured CAT activity with or without Dox treatment. Expression from the c-*jun*, c-*fos*, and *zif268* promoters was markedly increased by Dox, whereas the collagenase promoter (−73Col-CAT) was unresponsive to Dox (Fig. 8A). We next tested whether Dox inducibility is dependent upon the regulatory sequences responsible for the growth factor-induced expression of immediate-early genes. Both SRE- and CRE-CAT were highly responsive to Dox treatment. Given that SRE and CRE are important regulatory elements for c-*fos* expression (16), and that the *zif268* promoter contains several functional SREs (12), it is plausible that induction of c-*fos* and *zif268* by Dox may be mediated through these sites. On the other hand, TRE was not responsive to Dox. TRE is a
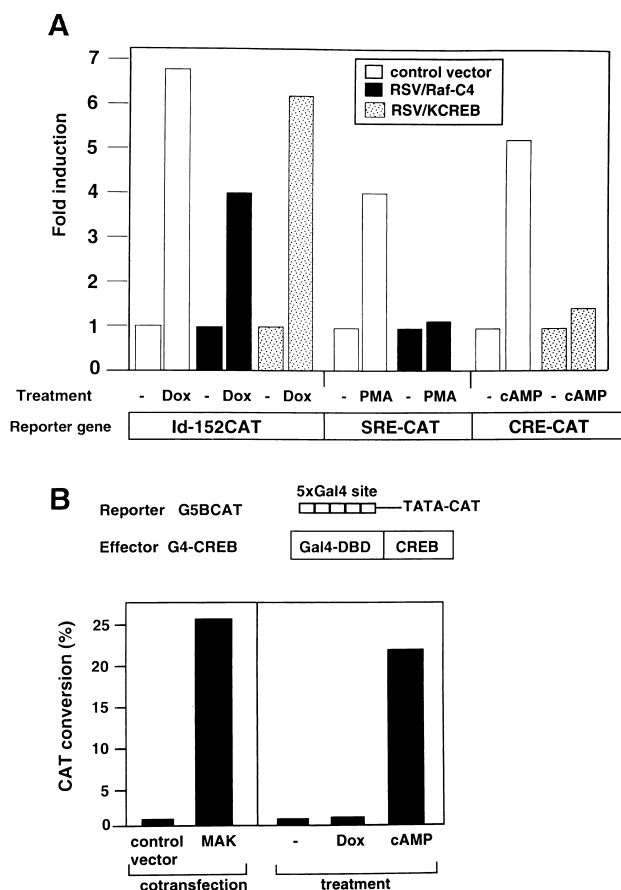

FIG. 7. The Id2A promoter is activated by Dox through Raf-1- and CREB-independent pathways. (A) Effects of dominant negative mutants of Raf-1 and CREB on Dox induction of the Id2A promoter. HeLa cells were transfected with 10 μg of either Id-152CAT, SRE-CAT, or CRE-CAT. Cells were cotransfected with 5 μg of expression vector encoding either a dominant negative mutant of Raf-1 (RSV/Raf-C4) or CREB (RSV/KCREB). Blank expression vector pRC/RSV was used as a control vector. At 20 h posttransfection, cells either were left untreated or were treated with Dox (1 μM), PMA (100 ng/ml), or cAMP (1 mM) for 20 h. CAT activities are expressed as the fold increase over activity without Dox treatment and are representative of three independent experiments. (B) Effects of Dox on transactivation of the Gal4-CREB fusion protein. At the top is a diagram of the reporter (G5BCAT) and effector plasmids (G4-CREB) used in the transactivation experiments. At the bottom is a graph showing CAT activities. HeLa cells were cotransfected with 10 μg of G5BCAT and 5 μg of G4-CREB, along with either 5 μg of blank expression vector (control vector) or expression vector encoding mouse PKA (MAK). At 20 h, some cells either were left untreated or were treated with Dox (1 μM) or cAMP (1 mM). At 40 h posttransfection, the cells were harvested and CAT activities were measured. The data presented are representative of three independent experiments.
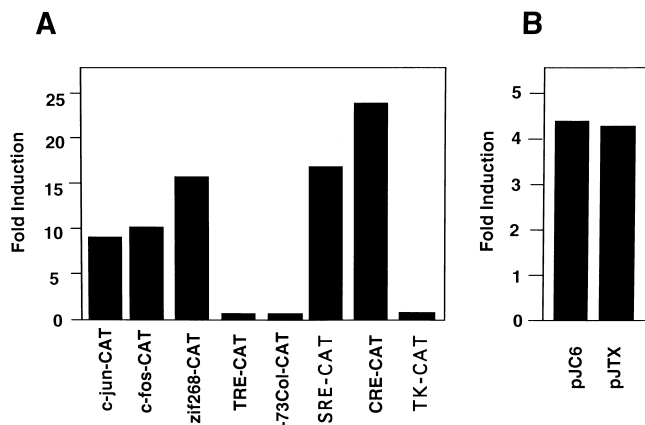
A

B

FIG. 8. Effects of Dox on the activity of immediate-early gene promoters. (A) HeLa cells were transfected with 10 μg of the indicated CAT constructs. At 20 h posttransfection, the cells either were left untreated or were treated with 1 μM Dox for 20 h. CAT activities were measured and are expressed as fold increase over the level of activity of each construct without treatment. (B) HeLa cells were transfected with 10 μg of either pJC6 or pJTX. CAT activities are expressed as the fold increase over the activity without Dox treatment and are representative of three independent experiments.

cis element responsible for induction of collagenase gene transcription (1). It is likely that the inability of −73Col-CAT to respond to Dox is due to the unresponsiveness of TRE to Dox. As a control, CAT activities from −73Col-CAT and TRE-CAT were tested; they were found to be markedly enhanced by PMA treatment, as expected (data not shown).

The observation that the c-*jun* promoter is responsive to Dox even though TRE-CAT is not Dox responsive is intriguing, because the c-*jun* promoter contains a functional TRE (2). To investigate whether this TRE is involved in induction of the c-*jun* promoter by Dox, we examined the effect of TRE mutation in the *jun* promoter on Dox inducibility. pJTX, which contains TRE mutations and is not TPA inducible, remains inducible by Dox at a level comparable to that induced by the wild-type construct pJC6 (Fig. 8B). Identical results were obtained when we used the constructs −132/+170*jun*CAT and −132/+170ΔTRE*jun*CAT, which contain the c-*jun* promoter region between −132 and +170 without or with TRE mutation, respectively (data not shown). Taken together, these results indicate that Dox induces immediate-early genes, including c-*jun*, in a manner independent of the TRE. These observations provide additional evidence that Dox-induced signals are distinct from those elicited by TPA.

DISCUSSION

***cis*-regulatory elements responsible for the induction of Id2A promoter activity by Dox.** In this study, we have delineated regulatory elements of the Id2A gene which are responsive to Dox. Several lines of evidence support the conclusion that IdATF sites located at −95 and −107 constitute the primary sequence requirements for Dox induction and that the integrity of these sites is essential for full induction by Dox. (i) Deletion analyses placed the 5′ boundary for the essential sequence for strong induction between −112 and −92 (Fig. 2). (ii) Site-specific-mutation analyses showed that individual mutation of the proximal and distal IdATF sites reduces the level of induction, although mutation of the proximal site has a more noticeable effect than mutation of the distal site (Fig. 3). (iii) Deletion of both sites completely abrogates inducibility (Fig.

3). (iv) A DNA fragment carrying these elements confers Dox inducibility on a heterologous promoter (Fig. 4).

Although the ATF site and CRE were originally identified as the response elements for adenovirus transactivator E1A protein (40) and cAMP (45), respectively, they have been found in the promoters or enhancers of many different genes which are not known to be regulated by either E1A or cAMP (21). A rapidly growing number of reports indicate that ATF/CRE sites are involved in a variety of signals, including human T-cell leukemia virus type 1 *tax* and cytokines (32, 56). Notably, precedence for the involvement of the ATF/CRE site in response to DNA-damaging agents is provided by studies of the polymerase β promoter, in which the DNA-damaging agent *N*-methyl-*N*′-nitro-nitrosoguanidine activates a consensus ATF/CRE site (33), but detailed mechanisms of this response beyond protein binding to the site are unknown.

When we compared human and mouse Id2A gene nucleotide sequences, we found conserved sequences similar to one IdATF site in the mouse Id2 gene promoter at the same position relative to the transcriptional start site (58). This evolutionary conservation suggests that the IdATF site plays an important role in the regulation of Id2 expression. In fact, mutation of this site resulted in the loss of 95% of the activity of the Id2 promoter in C2 myogenic cells, and binding of nuclear proteins to this site is down regulated during myogenic differentiation and is rapidly reactivated by serum (35). In this respect, ATF/CRE-like sites may play a role in regulating Id2 gene expression during cell differentiation as well as inducing expression in response to extracellular stimuli such as Dox and serum growth factors.

Signal transduction pathways elicited by Dox. The facts that the potent protein kinase inhibitor H7 abolished the Dox effect and that IdATF site binding activity is not significantly changed by Dox treatment lead us to suggest that Dox activates a kinase cascade that potentiates the activity of a factor already bound to the DNA. In this regard one could hypothesize that the IdATF site-binding protein is subjected to a mode of regulation by phosphorylation similar to that so well documented for CREB. In the case of CREB the catalytic subunit of PKA phosphorylates a serine residue located in CREB's transactivation domain and activates its transactivating function (17, 68). It has been postulated that phosphorylation increases the affinity of the transactivation domain for the basal transcription machinery or cofactor that mediates its interaction with the initiation complex (28). Clearly, in the case of Dox-induced transcription, alternative mechanisms, including effects on auxiliary factors, might be operating.

Perhaps the most interesting feature to emerge from this study is the finding of an apparently unique genetic response to Dox. We provided several lines of evidence suggesting that the major genetic response to Dox is independent of pathways involving either PKC or PKA. First, a cellular response that is not strongly inhibited by calphostin C and staurosporine is unlikely to depend on the PKC pathway. Second, Dox had no effect on the expression of TRE-CAT. These results indicate that Dox's effects are distinct from that of TPA. Third, results of cotransfection of RSV/Raf-C4 and RSV/KCREB suggest that Raf-1 and CREB do not transduce the major signals elicited by Dox. Finally, unlike cAMP and PKA, Dox cannot induce Gal4-dependent transcription by a Gal4-CREB chimeric protein. These data allow us to conclude that Dox triggers signal transduction pathways distinct from those elicited by PMA or cAMP stimulation. Such findings are compatible with previous studies in which Dox has been shown not to activate PKC (23), although the issues regarding the effects of anthracyclines on the activity of PKC remain controversial

(52). It is worth recalling that the TRE is one of the major target sequences for the classic UV response elicited by DNA-damaging agents (13, 53). Thus, we conclude that the genetic response to Dox is distinct from the UV response, although the specific signaling mechanisms remain to be discovered.

Our observations that AP-1 is not activated despite the activation of the c-*jun* and c-*fos* genes by Dox is somewhat surprising. However, many biochemical steps are required for c-Jun and c-Fos to form an active AP-1 complex, and at least three possible explanations for our observation, which are not mutually exclusive, can be envisaged. First, the signal transduction pathways activated by Dox may not lead to the proper phosphorylation and/or dephosphorylation of specific Ser or Thr residues that play crucial roles in AP-1's transcription activation function (25). Second, Dox may neither release nor inactivate a putative inhibitor of c-Jun which may be a prerequisite for the activation of c-Jun's transcription function (6). Third, it is also conceivable that Dox may induce the expression of proteins that are capable of inhibiting AP-1 activity. In this regard, it is intriguing to speculate that Dox induces oncoprotein E1a-like activity, because E1a has been shown to inhibit the function of AP-1 despite the fact that it transactivates c-*jun* and c-*fos* gene expression (19, 50, 64).

What the data presented here do emphasize is that one or more kinases, sensitive to H7 but distinct from PKC and PKA, are involved in the activation of Id2A transcription. Given that Dox inhibits cell proliferation and causes G2 delay (42), an important possibility is that Ser or Thr kinases involved in cell growth and cell cycle progression, such as cyclin-dependent kinase, casein kinase II, or DNA-activated protein kinase, may be involved in Dox-induced signaling pathways.

Factors involved in Dox-inducible gene expression. Although Dox activates transcription of CRE-CAT, three independent observations suggest that CREB itself is not a target of Dox's effect. First, antibodies to CREB or ATF-1 do not perturb the nuclear protein complex that binds to IdATF (Fig. 5). Second, Dox induction was not inhibited by cotransfection of a dominant repressor of CREB (KCREB) (Fig. 7A). Finally, the ability of the Gal4-CREB fusion protein (G4-CREB) to activate G5BCAT was not induced by Dox (Fig. 7B). A growing number of transcription factors belonging to the ATF/CREB family have been isolated (21), and the emerging picture of transcriptional regulation through members of this family has become more complicated than previously thought. Despite the similarity of their DNA binding domains, ATF proteins are highly divergent outside of the DNA binding domain (21). Moreover, ATF members can form heterodimers with other members of the ATF family or the AP-1/Jun family and thus can generate further diversity in mediating different transcription responses (20, 54). Our data argue against the possibility of the participation of either CREB or ATF-1 in DNA-protein complex formation at the IdATF site.

Furthermore, it is unlikely that formation of the C-I and C-II complexes involves ATF-2, ATF-3, and ATF-4, since antibodies directed against each of these factors fail to affect the C-I and C-II complexes (data not shown). In another set of experiments we attempted to determine whether Dox activates the transactivation function of ATF2. We cotransfected a reporter construct (Gal4 binding sites linked to the CAT gene) with a chimeric construct expressing the Gal4-ATF2 fusion protein and failed to find any evidence that Gal4-ATF2 activated Gal4-CAT expression in response to Dox (data not shown). These results allow us to exclude the possibility that ATF-2, -3, or -4 is involved in Dox-induced expression.

Despite the specific competition by an AP-1 oligonucleotide for the formation of complexes C-I and C-II (Fig. 5A), we can also exclude the possibility that AP-1 mediates the induction of the Id2A promoter by Dox, because TRE-CAT is unresponsive to Dox and antisera against c-Jun and c-Fos do not perturb the C-I and C-II complexes. However, on the basis of the fact that both bona fide ATF/CRE and AP-1 oligonucleotides efficiently compete with the IdATF site for binding of nuclear factors, we propose that members of the ATF- or AP-1/Jun family of transcription factors that are not targets for the cAMP- or PMA-induced signal transduction pathway are likely to be involved in Dox-induced signaling pathways.

Given that the c-*jun* promoter contains a functional RSRF site (related to serum response factor) at −59 (22), and that an SRE site is highly responsive to Dox, it will also be interesting to determine whether RSRF or SRF is involved in the Dox-induced activation of immediate-early genes.

The physiological role of the IdATF site and its cognate binding proteins is not understood. However, in light of the fact that the IdATF site serves as an efficient target for Dox's effects and that the IdATF site is evolutionarily conserved between human and mouse DNA, it is likely that IdATF site-binding proteins play an important role in cellular physiology. Since Id is a negative regulator of cellular differentiation, the pathways involved in its induction by such nuclear proteins are in essence repressors of tissue-specific gene expression and differentiation. Characterization of the relevant IdATF site-binding proteins will provide an opportunity to determine the signaling mechanisms triggered by Dox and to uncover the novel genetic response to Dox. Understanding the molecular and genetic events triggered by Dox would provide insights into how Dox exerts its effects on the transcriptional process and perturbs cellular integrity.

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